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(54) Title: NUCLEIC ACID MOLECULES FROM WHEAT, TRANSGENIC PLANT CELLS AND PLANTS AND THE USE  
THEREOF FOR THE PRODUCTION OF MODIFIED STARCH

(57) Abstract: Nucleic acid molecules are described encoding an R1-protein from wheat and methods and recombinant DNA  
molecules for the production of transgenic plant cells and plants synthesizing a modified starch. Additionally, the plant cells and  
plants resulting from those methods as well as the starch obtainable therefrom are described.

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Nucleic acid molecules from wheat, transgenic plant cells and plants and the use thereof for the production of modified starch

#### Description

The present invention relates to nucleic acid molecules encoding R1-protein from wheat and derivatives and parts thereof, said R1-protein, processes for the production of said R1-protein, transgenic plant cells and plants comprising said nucleic acid molecules, the transgenic plant cells and plants comprising said nucleic acid molecule, and the modified starch obtainable from said transgenic plant cells and plants.

The polysaccharide starch constitutes one of the most important storage substances in plants. Starch is widely used for the production of foodstuffs and plays also a significant role as a regenerative raw material in manufacturing of industrial products. In order to use starches in many different technical areas a large variety of optionally modified starches is required in order to meet the varying needs of the processing industry.

Although starch consists of a chemically homogeneous basic component, namely glucose, it does not constitute a homogeneous raw material. It is a complex mixture of molecules which differ in their degree of polymerization and degree of branching of the glucose chains: Amylose-type starch is a basically unbranched polymer consisting of  $\alpha$ -1,4-glycosidically branched glucose molecules, whereas amylopectin-type starch is a mixture of branched glucose chains, comprising additionally  $\alpha$ -1,6-glycosidic interlinkings.

The molecular structure of starch mainly depends on its degree of branching, the amylose/amylopectin ratio, the average chain-length, chain length distribution, and degree of phosphorylation, further determining the functional properties of the starch

and the aqueous solutions thereof. Important functional properties of the starch, resp., the aqueous solutions thereof are, e.g., solubility, tendency to retrogradation, capability of film formation, viscosity, pastification (binding and gluing) properties, and cold resistance. Additionally, the size of the starch granules may also determine the suitability of the starch for particular applications.

Since starch is often adapted by chemical and/or physical modification in order to meet the requirements of industry, there is a great need for the provision of modified starches which would render plant cells or plant parts containing modified starch more suitable for industrial processing, e.g., the production of foodstuff or technical products. Therefore, it is desired to avoid chemical and/or physical modification, which is time-consuming and expensive and to provide plants which synthesize a starch which meets more closely the demands of the starch processing industry.

Conventional methods for the preparation of modified plants which produce modified products, e.g., by classical breeding and/or the production of mutants, are limited to the use of homologous genes and are not always satisfying. Particularly in wheat, it is difficult to prepare a stable mutant by classical breeding due to the polyploidy of wheat (tetra- or hexaploidy). However, a wheat mutant producing waxy-type starch (amylose-free starch) was recently achieved by breeding methods (Nakamura et al., Mol. Gen. Genet. 248 (1995), 253-259).

A further alternative is the preparation of transgenic plants which comprise nucleic acid molecules suitable to modify plant starch metabolism in order to synthesize a modified starch. Such plants are produced by means of recombinant molecular biological techniques and the introduction of homologous and/or heterologous nucleic acid molecules (e.g., coding regions, regulatory elements, introns), which interfere in starch metabolism. However, the application of recombinant molecular biological techniques requires the availability of suitable nucleic acid which participate directly or indirectly (e.g., cosuppression, anti-sense-technology, generation of protein or ribozyme) in starch metabolism or starch biosynthesis (i.e.,

synthesis, modification and/or degradation of starch) with respect to quantity and/or quality of the starch.

Numerous genes are involved in starch metabolism. Therefore, a large number of genes encoding, e.g., branching enzymes, debranching enzymes, isoamylases, starch synthetases, ADP-glucose-pyrophosphorylases, have been used to modify starch metabolism in plants.

R1 proteins are involved in starch metabolism, especially with respect to the degree of phosphorylation of the starch and therefore, suitable to modify starch synthesis. In particular, R1-proteins and genes encoding R1-proteins derived from a number of plant species are known, i.e., potato from WO 97/11188-A1 and Lorberth et al., Nature Biotechnology 16 (1998), 473-477), maize from WO 98/27212-A1, rice from Sakaki et al., EMBL database entry Accession No. C 71741 (1997-09-19), and arabidopsis, ginger, mosses, cattail (*Typha latifolia*), and soybean from WO 99/53072-A1.

However, the presence of an R1-protein in wheat plants was not shown, corresponding nucleic acid molecules were not identified. Furthermore, the known nucleic acid molecules encoding R1-proteins are not always satisfying or suitable for the genetic engineering or the in vivo mutagenesis of wheat plants in order to modify wheat starch biosynthesis and/or metabolism.

Therefore, the problem to be solved by the present invention is to provide nucleic acid molecules encoding R1-protein derived from wheat and methods which allow the modification of starch metabolism in plants, especially in wheat plants in order to provide a modified starch, which differs from starch naturally synthesized with respect to its physical and/or chemical properties, especially wheat starch, exhibiting improved features, in particular for application in food and/or non-food industry.

These problems are solved by the embodiments of the present invention as claimed.

Therefore, the present invention relates to nucleic acid molecules encoding R1-protein comprising an amino acid sequence according to Seq. ID No. 2 and Seq. ID No. 9 or derivative or part thereof according to the cDNA insert of plasmid pTaR1-11 (DSM No. 12810) and plasmid RS26-88 (DSM No. 13511). Said R1-protein of the invention is involved in starch metabolism and is involved directly or indirectly in starch biosynthesis of wheat with respect to the degree of phosphorylation.

Within the meaning of the present invention, the term "derivative" regarding the R1-protein (polypeptide, amino acid sequence) of the invention encompasses a polypeptide derived from Seq ID No. 2 comprising about at least 60-79 amino acid radicals, preferably at least 80, more preferred at least 90, in particular at least 100, and most preferably about 101-111 amino acid radicals selected from the group of amino acid radicals consisting of 1E, 2V, 3V, 5G, 6L, 7G, 8E, 9T, 10L, 11V, 12G, 13A, 14Y, 15P, 16G, 17R, 18A, 20S, 21F, 23C, 24K, 25K, 27D, 28L, 30S, 31P, 34L, 35G, 36Y, 37P, 38S, 39K, 40P, 41I, 42G, 43L, 44F, 45I, 48S, 49I, 50I, 51F, 52R, 53S, 54D, 55S, 56N, 57G, 58E, 59D, 60L, 61E, 62G, 63Y, 64A, 65G, 66A, 67G, 68L, 69Y, 70D, 71S, 72V, 73P, 74M, 75D, 77E, 80V, 81V, 83D, 84Y, 87D, 88P, 89L, 90I, 92D, 95F, 96R, 99I, 100L, 101S, 103I, 104A, 105R, 106A, 107G, 108H, 109A, 110I, 111E, 112E, 113L, 114Y, 115G, 116S, 117P, 118Q, 119D, 121E, 122G, 123V, 124V, 126D, 127G, 128K, 129I, 130Y, 131V, 132V, 133Q, and 134T and comprising at least 1, preferably 2, and more preferred 3 of the amino acid radicals selected from the group consisting of 76V, 93S, and 97N of the amino acid radicals (hereinbefore indicated by single letter code) as specified in Seq ID No. 2.

Within the meaning of the present invention, the term "part" regarding the R1-protein (polypeptide, amino acid sequence) of the invention encompasses a poly- or oligopeptide consisting of about at least 10-19, preferably at least 20, more preferably at least 40, in particular preferably at least 80, and most preferably about 100-140 of the amino acid radicals of the R1-protein or derivative thereof according to the invention.

The present invention further relates to nucleic acid molecules comprising a nucleic acid molecule derived from Seq. ID No. 1 and Seq. ID No. 9 or derivatives or parts thereof, the 672 bp EcoR I/Kpn I insert of plasmid pTa R1-11 (DSM No. 12810) or derivatives or parts thereof, in particular the coding region (nucleotides 1 - 449) of Seq. ID No. 1 or derivatives or parts thereof, especially the coding region of the insert of plasmid pTa R1-11 (DSM No. 12810) and the coding region of plasmid RS26-88 (DSM No. 13511) or derivatives or parts thereof.

Within the meaning of the present invention, the term "derivative" regarding the nucleic acid molecule (nucleotide sequence, or polynucleotide) of the invention encompasses a polynucleotide comprising about at least 150-209 nucleotides, preferably at least 210, more preferred at least 240, in particular at least 270, and most preferably about 280-294 nucleotides selected from the group of nucleotides consisting of

- (a) 1C, 3G, 4A, 6G, 7T, 8G, 9G, 10T, 12A, 15G, 16G, 18C, 19T, 20T, 21G, 22G, 24G, 25A, 27A, 28C, 30C, 31T, 33G, 34T, 36G, 37G, 38A, 39G, 40C, 42T, 43A, 44T, 45C, 46C, 48G, 49G, 51C, 52G, 53T, 54G, 55C, 58T, 59G, 60A, 61G, 63T, 64T, 67T, 69T, 70G, 72A, 73A, 75A, 76A, 77A, 79A, 81G, 82A, 84C, 85T, 88A, 89C, 90T, 91C, 92T, 93C, 94C, 97A, 100T, 103T, 105G, 106G, 107T, 108T, 109A, 110C, 111C, 112C, 114A, 115G, 116C, 117A, 118A, 120C, 121C, 123A, 124T, 126G, 127G, 129C, 130T, 132T, 133T, 134C, 135A, 136T, 137A, 138A, 144T, 145C, 147A, 148T, 149C, 150A, 151T, 152C, 153T, 154T, 155C, 156C, 157G, 159T, 160C, 162G, 163A, 165T, 166C, 168A, 169A, 171G, 172G, 174G, 175A, 177G, 178A, 181T, 182G, 183G, 184A, 185A, 186G, 187G, 188T, 189T, 190A, 192G, 193C, 195G, 196G, 198G, 199C, 201G, 202G, 205T, 207T, 208A, 210G, 211A, 213A, 214G, 215T, 216G, 217T, 219C, 220C, 222A, 223T, 224G, 225G, 226A, 227T, 228G, 230G, 231G, 232A, 234G, 235A, 238A, 240G, 241T, 242T, 243G, 244T, 245A, 247T, 249G, 250A, 252T, 253A, 256C, 259C, 261G, 262A, 263C, 264C, 265C, 268T, 270A, 271T, 276G, 277A, 281T, 285T, 286T, 287C, 288C, 289G, 295C, 296A, 297A, 298T, 299C, 300C, 301T, 303T, 304C,



306A, 308C, 309A, 310T, 312G, 313C, 315C, 316G, 318G, 319C, 320T, 321G, 322G, 324C, 325A, 327G, 328C, 330A, 331T, 332C, 333G, 334A, 335G, 336G, 337A, 338G, 339C, 340T, 342T, 343A, 344T, 345G, 346G, 348T, 349C, 351C, 352C, 354C, 355A, 357G, 358A, 361T, 363G, 364A, 365G, 366G, 367G, 369G, 370T, 371A, 372G, 373T, 374G, 375A, 377G, 378G, 379A, 380T, 381G, 382G, 384A, 385A, 387A, 388T, 390T, 391A, 393G, 394T, 396G, 397T, 399C, 400A, 401G, 402A, 403C, 404A, 406A, 407C, 408C, 409A, 410C, 411A, 412G, 413A, 414T, 415G, 416T, and 419T as specified in Seq. ID No. 1, and

- (b) comprising about at least 15-19 nucleotides, preferably at least 20, more preferred at least 25, in particular at least 27, and most preferably about 30-32 nucleotides selected from the group consisting of  
17A, 23A, 50C, 56C, 65C, 68G, 71T, 104G, 113T, 143G, 159C, 161A, 167T, 191C, 203G, 206G, 218C, 221T, 229T, 233A, 248C, 251C, 257G, 269C, 272C, 279T, 280C, 290G, 326C, 341C, 347G, 350A, 353A, 359T, 383G, 392C, and 405T as specified in Seq. ID No. 1.

It is expressly stated that the numbering of the elements of the sequences (on one hand nucleic acid sequence and on the other amino acid sequence) shall not be understood as a fixed or limiting definition. The numbering shall merely provide the information of the positions of the sequence elements to each other in relative terms and is therefore a reference.

Furthermore, the term "derivative" regarding the nucleic acid molecule encoding an R1-protein according to the invention encompasses a nucleic acid molecule which is different from Seq. ID No. 1 and/or Seq ID No. 9 by addition, deletion, insertion or recombination of one or more nucleotides and fulfills the definition as given above under (b).

Additionally, the term „derivative“ regarding the nucleic acid molecule encoding an R1-protein according to instant invention encompasses a complementary or reverse complementary polynucleotide of the nucleic acid molecule according to the

invention or parts thereof. Furthermore, the term „derivative“ regarding the nucleic acid molecule encoding an R1-protein according to instant invention comprises a polynucleotide hybridizing with the nucleic acid molecule according to the invention or parts thereof, which fulfills the definition as given above under (b).

The term “hybridization” denotes, for the purposes of the present invention, a hybridization under conventional hybridization conditions, preferably under stringent conditions as described, for example, by Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Especially preferably, “specific hybridization” means the following conditions:

Hybridization buffer: 2 x SSC; 10 x Denhardt solution (Ficoll 400 + PEG + BSA; ratio 1:1:1); 0.1% SDS; 5 mM EDTA; 50 mM Na<sub>2</sub>HPO<sub>4</sub>; 250 µg/ml herring sperm DNA; 50 µg/ml tRNA; or 0.25 M sodium phosphate buffer pH 7.2; 1 mM EDTA; 7% SDS at a  
Hybridization temperature of      T = 55 to 68 °C,  
Wash buffer :                              0.2 x SSC; 0.1% SDS and  
Wash temperature:                        T = 40 to 68 °C.

The molecules which hybridize with the nucleic acid molecules according to the invention or with the nucleic acid molecules to be suitably employed according to the invention also encompass parts, derivatives and allelic variants of the nucleic acid molecules according to the invention or the nucleic acid molecule to be suitably employed in accordance with the invention.

The term “derivative” means, within the context of the present invention, that the sequences of these molecules differ from the sequences of the nucleic acid molecules according to the invention or to be suitably employed in accordance with the invention in one or more positions and exhibit a high degree of homology to these sequences. Homology means a sequential identity of at least 60%, preferably



over 70%, and especially preferably over 85%, in particular over 90% and very especially preferably over 95%. The deviations relative to the nucleic acid molecules according to the invention or to the nucleic acid molecules to be suitably employed in accordance with the invention may have originated by means of one or more deletions, substitutions, insertions (addition) or recombinations.

Furthermore, homology means that a functional and/or structural equivalence exists between the nucleic acid molecules in question and the proteins encoded by them. The nucleic acid molecules which are homologous to the molecules according to the invention or to the molecules to be suitably employed in accordance with the invention and which constitute derivatives of these molecules are, as a rule, variations of these molecules which constitute modifications which exert the same, a virtually identical or a similar biological function. They may be naturally occurring variations, for example sequences from other plant species, or mutations, it being possible for these mutations to have occurred naturally or to have been introduced by directed mutagenesis. The variations may further be synthetic sequences. The allelic variants may be naturally occurring variants or else synthetic variants or variants generated by recombinant DNA technology.

The term "part" regarding the nucleic acid molecule encoding an R1-protein according to instant invention encompasses a poly- or oligonucleotide consisting of about at least 30-99, preferably at least 100, more preferably at least 200, in particular at least 300, and most preferably at least 400 of the nucleotides of the nucleic acid molecule encoding an R1-protein or derivative thereof according to the invention. The term „part“ is not limited to portions of the nucleic acid molecules which are long enough to encode a functionally active portion of the R1-protein as described.

In a preferred embodiment of instant invention, the terms "derivative" and/or "part" according to instant invention encompass a polynucleotide, resp., poly- or

oligopeptide as defined above, which exhibits functional and/or structural equivalence of the R1-gene (i.e., the nucleic acid molecule encoding R1-protein), resp., R1-protein derived from wheat. The term "functional and/or structural equivalence" generally means the same, an equivalent or a similar function of the resp. molecule of the invention, especially biological function. The term „part“ is, however, not limited to portions of the said nucleic acid molecule, which are sufficient to encode a functionally active portion of the said protein.

The R1-proteins encoded by the nucleic acid molecules according to the invention may exhibit certain common characteristics, e.g., enzyme activity, molecular weight, immunologic reactivity, conformation, mobility in gel electrophoresis, chromatographic characteristics, sedimentation coefficients, solubility, spectroscopic properties, stability, pH-optimum and/or temperature-optimum of the enzymatic activity, etc.

The nucleic acid molecule of the invention may be isolated from, e.g., natural sources, prepared by methods of genetic engineering or molecular biology (e.g., PCR) or produced by means of chemical synthesis. The nucleic acid molecule of the invention is preferably a DNA or RNA molecule, e.g., a cDNA or genomic DNA molecule. Optionally, the nucleic acid molecule of the invention comprises one or more intervening sequences (introns).

In another preferred embodiment the nucleic acid molecule of the invention comprises one or more regulatory elements that ensure the transcription and synthesis of an RNA molecule in a prokaryotic and/or eukaryotic cell, preferably in a plant cell.

The nucleic acid molecule according to the invention is suitable in order to modify starch biosynthesis/metabolism in a cell, preferably in a plant cell by means of sense expression of the nucleic acid molecules of the invention, antisense expression of the nucleic acid molecules of the invention, expression of a suitable ribozyme,

cosuppression or in vivo mutagenesis.

Therefore, the invention relates also to the use of the nucleic acid molecule of the invention, in particular a DNA molecule, which allows the synthesis of a translatable or a non-translatable mRNA molecule (sense- or anti-sense-, co-suppression effect or ribozyme) in a cell or a plant cell which modifies the R1-protein expression level.

Generally, the use of the nucleic acid molecules of the invention is suitable in any plant species. However, monocotyledonous and dicotyledonous plants are preferred, in particular crop plants and most preferred starch-storing plants, e.g., rye, barley, oats, wheat, millet, sago, rice, maize, peas, wrinkled peas, cassava, potato, tomato, oilseed rape, soy bean, hemp, flax, sunflower, cow-pea, arrowroot, clover, ryegrass, or alfalfa, in particularly potato, maize, rice or wheat plants.

The method of co-suppression is well known to the person skilled in the art (Jorgensen, Trends Biotechnol. 8 (1990), 340-344, Niebel et al., Curr. Top. Microbiol. Immunol. 197 (1995), 91-103, Flavell et al., Curr. Top. Microbiol. Immunol. 197 (1995), 43-56, Palaqui & Vaucheret, Plant. Mol. Biol. 29 (1995), 149-159. Vaucheret et al., Mol. Gen. Genet. 248 (1995), 311-317 and de Borne et al., Mol. Gen. Genet 243 (1994), 613-621.

In a further embodiment the present invention relates to a DNA molecule encoding an RNA molecule exhibiting ribozyme activity which specifically cleaves transcripts of the DNA molecule of the invention. Ribozymes are catalytically active RNA molecules capable of cleaving RNA molecules and specific target sequences. By means of recombinant DNA techniques it is possible to determine the specificity of a ribozyme with respect to the nucleic acid molecule of the invention. In order to prepare a DNA molecule encoding a ribozyme which specifically cleaves a transcript of a DNA molecule of the invention, e.g., a DNA sequence (DNA molecule) encoding a catalytic domain of a ribozyme is bilaterally linked to a DNA sequence of the invention. A nucleic acid sequence encoding the catalytic domain is, e.g., the

catalytic domain of the satellite DNA of the SCMo virus (Davies et al., Virology 177 (1990), 216-224) or the satellite DNA of the TobR virus (Steinecke et al., EMBO J. 11 (1992), 1525-1530; Haseloff and Gerlach, Nature 334 (1988), 585-591). The DNA sequence flanking the catalytic domain is preferably the DNA molecule of the invention or part thereof, which shall serve as a target. The general principle of the expression of ribozymes and the method is described in EP-B1 0 321 201. The expression of ribozymes in plant cells is further described in Feyter et al. (Mol. Gen. Genetic. 250 (1996), 329-338).

A reduction of the activity of the R1-protein of the invention in a plant cell can also be achieved by the method of "in vivo mutagenesis". Hereby, a hybrid RNA/DNA oligonucleotide (chimeroplast) is introduced into a cell (Kipp et al., Poster Session at the 5th International Congress of Plant Molecular Biology, September 21 to 27, 1997, Singapore; Dixon and Arntzen, meeting report on "Metabolic Engineering in Transgenic Plants" Keystone Symposia, Copper Mountain, CO, USA, TIBTECH 15 (1997), 441-447; WO 95/15972-A1; Kren et al., Hepatology 25 (1997), 1462-1468; Cole-Strauss et al., Science 273 (1996), 1386-1389).

Therefore, yet another object of the invention is a plant, preferably a wheat plant, exhibiting an altered activity of the R1-protein according to the invention obtainable by in vivo mutagenesis.

Furthermore, the invention relates to a vector, especially a plasmid, cosmid, virus, bacteriophage and the like, suitable in genetic engineering, comprising a nucleic acid molecule (e.g., DNA and/or RNA) of the invention, in particular a vector suitable in genetic engineering of bacteria and/or plants. The term "vector" means a suitable vehicle known to the skilled artisan, which allows the targeted transfer of single or double-stranded nucleic acid molecules into a host cell, e.g., a DNA or RNA virus or virus fragment, a plasmid which is suitable for the transfer of a nucleic acid molecule into a cell in the presence or absence of regulatory elements, metal particles as employed, e.g., in the particle-gun method, but also a nucleic acid molecule which

can be directly introduced into a cell by chemical and/or physical methods.

In a further embodiment the invention relates to a transgenic host cell, which is transformed and/or recombinantly manipulated by a nucleic acid molecule or a vector according to the invention, in particular a transgenic prokaryotic or eukaryotic cell, and more preferably a transgenic bacterial or a plant cell. The transgenic host cell according to the invention, especially the transgenic bacterial or plant cell contains one or more nucleic acid molecules of the invention, which are stably integrated into the genome of said cell, preferably not at the homologous genomic locus, resp., not at the location of the naturally occurring gene within the genome. The transgenic cells according to the invention may be identified by Southern Blot, Northern Blot and/or Western Blot analysis.

Additionally, the present invention relates to a transgenic cell, which is derived from the transgenic host cell of the invention and/or the descendants thereof containing a nucleic acid molecule or a vector according to the invention.

By provision of the nucleic acid molecule and/or the vector of the invention a transgenic plant cell or plant is prepared by means of recombinant DNA techniques comprising a nucleic acid molecule and/or a vector according to the invention, in particular a monocotyledonous or dicotyledonous plant cell or plant, preferably a crop plant cell or a plant, in particular a plant cell or plant selected from the group consisting of a potato, maize, oat, rye, barley, wheat, pea, rice, millet, wrinkled peas, cassava, sago, tomato, oilseed rape, soy bean, hemp, flax, sunflower, cow-pea, arrowroot, clover, ryegrass, alfalfa, and maniok.

The transgenic plant cell or plant of the invention synthesizes a modified starch which differs from the starch synthesized in a wildtype (non-transformed) plant with respect to structure and/or physical and/or chemical properties. By the methods of genetic engineering and/or molecular biology, a vector and/or a nucleic acid molecule of the invention is introduced into a plant cell, preferably linked to one or

more regulatory elements, which ensure transcription and/or translation in said plant cell. Optionally, the resulting transgenic plant cell is subsequently regenerated to a whole plant.

Therefore, the present invention relates to a transgenic plant cell, in particular a monocotyledonous or dicotyledonous plant cell, preferably, a potato, maize, oat, rye, barley, wheat, pea, rice, millet, wrinkled peas, cassava, sago, tomato, oilseed rape, soy bean, hemp, flax, sunflower, cow-pea, arrowroot, clover, ryegrass, alfalfa, or maniok cell, in particular potato, wheat, maize or rice cell, comprising a nucleic acid molecule and/or a vector according to the invention.

The invention relates also to a process for the preparation of a transgenic host cell, preferably, a plant cell comprising the step of introducing a nucleic acid molecule and/or a vector of the invention into the genome of a host cell which is a procaryotic or eucaryotic cell, preferably, into the genome of a plant cell. Preferably, said cell contains a nucleic acid molecule which is linked to one or more regulatory elements which ensure transcription and/or translation in said cell. Suitable regulatory elements are preferably homologous or heterologous with respect to the nucleic acid molecule of the invention.

In another embodiment, the invention relates to a transgenic plant cell wherein the presence of a (homologous or optionally, heterologous) nucleic acid molecule of the invention leads directly or indirectly to the expression of the R1-protein of the invention or, alternatively, to the inhibition of the expression of one or more endogenous genes encoding an R1-protein. Preferably, the transgenic plant cell comprises a nucleic acid molecule which is selected from the group consisting of:

(a) a nucleic acid molecule of the invention, preferably a DNA molecule, which is transcribed into sense-RNA, which leads to the expression of an R1-protein of the invention;

(b) a nucleic acid molecule of the invention, preferably a DNA molecule, which is transcribed into antisense-RNA which leads to the reduction (inhibition) of



the expression of one or more endogenous genes encoding an R1-protein;

(c) a nucleic acid molecule of the invention, preferably a DNA molecule, which is transcribed into a cosuppression-RNA (sense RNA) which leads to a reduction (inhibition) of the expression of one or more endogenous genes encoding an R1-protein;

(d) a nucleic acid molecule of the invention, preferably a DNA molecule, which is transcribed into a ribozyme which specifically cleaves a transcript of one or more endogenous genes encoding an R1-protein; and

(e) a nucleic acid molecule of the invention which is introduced by in vivo mutagenesis, which modifies one or more endogenous genes encoding an R1-protein,

hereby modifying starch metabolism/biosynthesis in said cell.

If the modification of starch metabolism in plants is achieved by means of an antisense effect, the DNA molecule of the invention is linked in antisense orientation with one or more regulatory elements ensuring the transcription and/or translation in a plant cell, optionally comprising one or more intron(s) of a corresponding genomic sequence of the polynucleotide to be expressed. The antisense RNA should exhibit a minimum of about 15-25 nucleotides, preferably at least about 50-100 nucleotides and most preferably at least about 200-1000 nucleotides.

In a further embodiment the decrease in the amount of an R1-protein in the transgenic plant cell is achieved by a ribozyme comprising a nucleic acid molecule of the invention. In order to express said ribozyme molecule in a transgenic plant cell of the invention, a DNA molecule encoding said ribozyme is linked to one or more regulatory elements which ensure transcription and/or translation.

By means of methods well known to the skilled person, the transgenic plant cell can be regenerated to a whole plant. The transgenic plant comprising a transgenic plant cell of the invention which is obtainable by regenerating the transgenic plant cell of the invention and the process for the preparation of said transgenic plant are also

subject-matter of the present invention.

The transgenic plant of the invention is a monocotyledonous or dicotyledonous plant, preferably a crop plant, in particular a rye, barley, oat, rice, wheat, millet, sago, maize, pea, wrinkled pea, cassava, potato, tomato, manioc, oil seed rape, soy bean, hemp, flax, sunflower, cow-pea, white clover, ryegrass, alfalfa or arrowroot plant, most preferred a maize, wheat, rice, or potato plant.

Further the present invention relates to the propagation material, seed, organs, and parts of the plants of the invention.

The present invention also relates to a process for the production of starch comprising the step of introducing a transgenic plant cell, plant and/or part of a plant according to the invention into a process for the production/extraction of starch.

The present invention further relates to a process for the production of modified starch comprising the step of introducing a starch according to the invention into a process of chemical and/or physical modification/treatment of starch.

Processes for starch extraction from plants, plant cells, or parts thereof are well known in the art. Such processes are described, for example, in Eckhoff et al. (Cereal Chem. 73 (1996), 54-57). Extraction of maize starch is achieved by, e.g., "wet-milling". Other methods for starch extraction from various plants are described, e.g., in Starch: Chemistry and Technology (eds.: Whistler, BeMiller and Paschall (1994) 2nd Edition, Academic Press Inc. London LTD; ISBN 0-12-746270-8; Chapter XII, page 417-468: Corn and Sorghum Starches: Production; by Watson, S.A.; Chapter XIII, page 469-479: Tapioca, Arrowroot and Sago Starches: Production by Corbishley and Miller; Chapter XIV, page 479-490: Potato Starch: Production and Uses; by Mitch; Chapter XV, page 491-506: Wheat starch: Production, Modification and Uses; by Knight and Olson; and Chapter XVI, page 507-528: Rice starch: Production and Uses; by Rohwer and Klem). Means usually

used in methods for the extraction of starches from plant materials are separators, decanters, hydroclones and different kinds of machines for drying the starch, e.g., spray drier or jet drier.

The present invention also relates to the modified starch obtainable from the transgenic plant cells, plants and/or parts of a plant of the invention, preferably from wheat. The transgenic cells or plants of the invention synthesize a modified starch which differs from a starch obtainable from non-transformed plants with respect to the degree of phosphorylation. In a specific embodiment of the invention, the starch according to the invention exhibits an increased phosphate content compared to a starch obtainable from corresponding non-transformed cells or plants. An increased phosphate content (phosphate-monoester content) means a starch containing about at least 10-30%, more preferably at least 30 %, even more preferably at least 50 %, and particularly preferred more than 100 % up to about 1000-5000 % increased phosphate content compared to the phosphate content of a starch obtainable from a corresponding non-transformed plant. In general, the percentage values refer to the glucose-6-phosphate (glu-6-P) content of wheat starch of about 0.3 nmol glu-6-P/mg starch determined, e.g., according to the method of Lim et al. *Cereal Chem.*, (1994) 71, 448. Accordingly, the wheat starch according to instant invention comprises a glucose-6-phosphate content of at least 0.4 nmol/mg starch, preferably of at least 0.6 nmol/mg, more preferred at least 0.8 nmol/mg, in particular at least 1.0 nmol/mg, especially at least 1.5 nmol/mg, and most preferred at least 3.0 nmol/mg starch.

In another embodiment of the invention the starch of the invention exhibits a decreased phosphate content (phosphate-monoester content) of about at least 5-20%, preferably about at least 21-50 %, even more preferably about 51-95 % decreased phosphate content compared to the phosphate content of a starch obtainable from a corresponding non-transformed plant. Accordingly, the wheat starch according to instant invention exhibits a glucose-6-phosphate content of less than 0.2 nmol/mg starch, preferably less than 0.1 nmol/mg, more preferred less than 0.05 nmol/mg, in particular less than 0.02 nmol/mg, especially less than 0.01

nmol/mg, and most preferred less than 0.001 nmol/mg starch.

Another object of the invention is a method for the preparation of the R1-protein of the invention or derivative or part thereof comprising the steps of cultivating a transgenic host cell of the invention under conditions allowing for the expression of said R1-protein or derivative or part thereof and isolating said R1-protein or derivative or part thereof from said cells and/or the cultivating medium of said cells.

Furthermore, the invention relates to an R1-protein (R1-polypeptide) or derivative or part thereof encoded by the nucleic acid molecule of the invention obtainable by the method for the production of an R1-protein or derivative or part thereof according to the invention, preferably an R1-protein or derivative or part thereof derived from wheat, especially according to Seq. ID No. 2 and/or Seq. ID No. 10.

Within the present invention, the term "regulatory element which ensures transcription and/or translation" preferably has the meaning of a nucleic acid molecule (e.g., DNA and/or RNA) which allows for the initiation and/or termination of transcription in a cell, such as promoters, enhancers, terminators etc.. The term "regulatory element which ensures transcription and/or translation" may also comprise a nucleic acid molecule which leads to a timely and/or locally (endosperm, root, tuber, leaf, stem, seed, fruit, apoplast, vacuole, cytosol, plastid, mitochondrion, lysosome) limited transcription within a plant/or plant cell or which is chemically inducible.

For the expression of the nucleic acid molecules of the invention in a plant cell any active promoter may be used. Said promoter may be homologous or heterologous with respect to the plant cell to be transformed, e.g., for constitutive expression the 35S promoter of the cauliflower mosaic virus (CaMV) (Odell et al., Nature 313 (1985), 810-812; Mitsuhashi et al., Plant and Cell Physiology 37 (1996), 49-59) or the promoter construct described in WO 94/01571-A1. Suitable are also promoters which lead to a locally and/or timely limited expression determined/induced by

endogenous and/or exogenous factors (e.g., WO 93/07279-A1), e.g., a limited expression with respect to a particular tissue or part of the plant (Stockhaus et al., EMBO J. 8 (1989), 2245-2251). Promoters which are active in the starch-storing part of the plant to be transformed are preferred. Preferred parts of plants are for the expression of the nucleic acid molecules of the invention, e.g., maize, wheat and rice grains or seeds and potato tubers and the like. For the transformation of potato the tuber-specific B33-promoter (Rocha-Sosa et al., EMBO J. 8 (1989), 23-29) may be used. Apart from promoters, DNA regions initiating transcription may also contain DNA sequences ensuring a further increase of transcription, such as the so-called enhancer-elements. For expression in plant cells, and in particular in wheat cells, the following promoters can be used: the 35S promoter (Odell et al. supra; Mitsuhashi et al., supra), the ubiquitin promoter (US 5,614,399, Christensen et al., Plant Mol. Biol. 18 (1992), 675-689; Takimoto et al., Plant Mol. Biol. 26 (1994), 1007-1012; Comejo et al., Plant Mol. Biol. 23 (1993), 567-581; Toki et al., Plant Phys. 100 (1992), 1503-1507), glutelin promoter (Leisy et al., Plant Mol. Biol. 14 (1990), 41-50; Zheng et al., Plant J. 4 (1993), 357-366; Kononowicz et al., Joint annual meeting of The American Society of Plant Physiologists and The Canadian Society of Plant Physiologists, Minneapolis, Minnesota, USA, July 1 to August 4, 1993, Plant Physiol. 102 (suppl.) (1993) 166; Zhao et al., Annual Meeting of the American Society of Plant Physiologists, Pittsburgh, Pennsylvania, USA, August 1 to 5, 1992. Plant Physiol. 99 (1 Suppl.) (1992), 85; Yoshihara et al., FEBS Lett. 383 (1996), 213-218, the actin promoter (McElroy et al., Plant Cell 2 (1990), 163-171), cab-6 promoter (Plant and Cell Physiology 35 (1994), 773-778), RTBV promoter (Yin et al., Plant J. 12 (1997), 1179-1188), CVMV promoter (Verdaguer et al., Plant Mol. Biol. 31 (1996), 1129-1139), rab 16B promoter (Plant Physiol. 112 (1996), 483-491), promoter of the psbD-C operon (To et al., Plant and Cell Physiology 37 (1996), 660-666), Tpi promoter (Snowden et al., Plant Mol. Biol. 31 (1996), 689-692), Osgrpl promoter (Xu et al., Plant Mol. Biol. 28 (1995), 455-471, Ltp2 promoter (Kalla et al., Plant J. 6 (1994), 849-860), ADH1 promoter (Kyoizuka et al., Mol. Gen. Genet. 228 (1991), 40-48) and LHCP promoter (EMBO J. 10 (1991), 1803-1808).



Furthermore, the term "regulatory element" also comprises a termination signal suitable to finalize the transcription and/or to add a poly-A-tail to the transcribed nucleic acid molecule. Examples for a termination signal are the 3'-nontranslatable regions comprising the polyadenylation signal of the nopaline synthase gene (NOS gene) or octopine synthase gene (Gielen et al., EMBO J. 8 (1989), 23-29) from agrobacteria, the 3'-nontranslatable region of the gene of the storage protein from soy bean or small subunit of ribulose-1,5-biphosphate-carboxylase (ssRUBISCO). Optionally, the term "regulatory element" comprises a nucleic acid molecule which ensures, e.g., the specific location of transcription and/or translation of the nucleic acid molecule of the invention in a specific tissue (e.g., endosperm, leaf, stem, tuber, meristem, fruit, root, seed) or cell compartment (e.g., cytosol, apoplast, plastid, mitochondrion, vacuole, lysosome). Optionally, the term "regulatory element" comprises also nucleic acid molecules which ensures, e.g., timely limited transcription and/or translation of the nucleic acid molecule of the invention. Furthermore, the "regulatory element" may optionally be chemically triggered.

The introduction of a nucleic acid molecule of the invention into a plant cell, preferably a DNA or RNA molecule, is generally carried out using cloning vectors which ensure stable integration of the nucleic acid molecule into the plant genome. In order to introduce a nucleic acid molecule into a higher plant a large number of cloning vectors are available containing a replication signal for *E. coli* and a marker gene for the selection of transformed bacterial cells, e.g., pBR322, pUC series, M13mp series, pACYC184. The nucleic acid molecule of the invention may be integrated into the vector at a suitable restriction site by use of one or more restriction endonuclease enzymes. The obtained plasmid is used for the transformation of, e.g., *E. coli* cells. Transformed cells are cultivated in a suitable medium and subsequently harvested and lysed, the plasmid DNA is recovered by means of standard methods and is generally analyzed by restriction and/or sequence analysis. After each manipulation the plasmid DNA may be cleaved and the obtained DNA fragments linked to other DNA sequences. In order to introduce DNA into a plant host cell a wide range of transformation methods and techniques



are available, e.g., T-DNA transformation by use of *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, fusion of protoplasts, injection of DNA, electroporation of DNA, and the introduction of DNA by membrane permeation (PEG) or means of the biolistic method and others. If whole plants are to be regenerated from transgenic plant cells, a selectable marker gene should be present. If the Ti- or Ri-plasmid is used, e.g., for transformation of the plant cell, at least the right border, preferably, the right and left border of the Ti- and Ri-plasmid T-DNA should be linked with the polynucleotide to be introduced into the plant cell as a flanking region.

If *Agrobacteria* are used for transformation, the DNA to be introduced should be cloned into either an intermediate vector or binary vector. Due to sequence homologies to the sequences of the T-DNA, the intermediate vectors may be integrated into the Ti- or Ri-plasmid of the *Agrobacterium* by homologous recombination. Said intermediate vectors also contain the vir-region necessary for the transfer of the T-DNA. Since intermediate vectors cannot replicate in *Agrobacteria*, a helper plasmid may transfer the intermediate vector to *Agrobacterium* (conjugation). Binary vectors may replicate in *E. coli* and in *Agrobacteria*. They contain a selectable marker gene and a linker or polylinker which is flanked by the right and the left T-DNA border region. They may be transformed directly into the *Agrobacteria* (Holsters et al. *Mol. Gen. Genet.* 163 (1978), 181-187). The plasmids used for the transformation of *Agrobacteria* further comprise a selectable marker gene, e.g., the NPT II gene which allows for the selection of the transformed bacteria. The plasmid may comprise further selection marker genes e.g. conferring resistance against spectinomycin (Svab et al., *Proc. Natl. Acad. Sci. U.S.A.* 87 (1990), 8526-8530; Sval et al., *Plant. Mol. Biol.* 14 (1990), 197-206), streptomycin (Jones et al., *Mol. Gen. Genet.* 91 (1987), 86-91; Svab et al., *Proc. Nat. Acad. Sci. U.S.A.* 87 (1990), 8526-8530; Svab et al., *Plant. Mol. Biol.* 14 (1990), 197-206), phosphinothricine (De Block et al., *EMBO J.* 6 (1987), 2513-2518), glyphosate (Thompson et al., *EMBO J.* 6 (1987), 2519-2523; Thompson et al., *Weed Sci.* 35 (1987), 19-23 (suppl.)), or hygromycin (Waldron et al., *Plant Mol. Biol.* 5 (1985), 103-108). The *Agrobacterium* host cell should contain a plasmid carrying a vir-region. The vir-region is necessary for the transfer of the T-DNA into the plant

cell. Additional T-DNA may be present. The transformed *Agrobacterium* is further used for the transformation of plant cells.

The use of T-DNA for the transformation of plant cells is described in EP-A-120 516; Hoekema, In: The Binary Plant Vector System Offsetdrukkerij Kanters B.V., Alblasterdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant. Sci., 4, 1-46 and An et al., EMBO J. 4 (1985), 277-287. Binary vectors are commercially available, e.g., pBIN19 (Clontech Laboratories, Inc., USA).

For transferring the DNA into the plant cells, plant explants may be co-cultivated with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. Whole plants may be regenerated from infected plant material (e.g., pieces of leaves, stem segments, roots, but also protoplasts or suspension-cultivated plant cells) in a suitable medium which allows for the selection of transformed cells (e.g., containing antibiotics or biocides etc.). The obtained plants are screened for the presence of the introduced DNA. Other possibilities in order to introduce foreign DNA by using, e.g., the biolistic method or by transforming protoplasts are known to the skilled person (e.g., Willmitzer, L., 1993 Transgenic plants. In Biotechnology, A Multi-Volume Comprehensive Treatise (H.J. Rehm, G. Reed, A. Pühler, P. Stadler, eds.), Vol. 2, 627-659, VCH Weinheim-New York-Basel-Cambridge).

The transformation of dicotyledonous plants by Ti-plasmid-vector systems by means of *Agrobacterium tumefaciens* is a well-established method. *Agrobacteria* can also be used for the transformation of monocotyledonous plants (Chan et al., Plant Mol. Biol. 22 (1993), 491-506; Hiei et al., Plant J. 6 (1994), 271-282).

Alternative methods for the transformation of monocotyledonous plants are, e.g., the transformation by means of the biolistic approach, protoplast transformation, electroporation of partially permeabilized cells, the introduction of DNA by means of glass fibers. Various references refer to the transformation of maize (WO 95/06128-A1, EP-A-0 513 849; EP-A-0 465 875). EP-A-292 435 describes a method how to obtain fertile plants starting from mucousless, friable granulous maize callus. Shillito

et al. (Bio/Technology 7 (1989), 581) started from callus-suspension cultures which produce dividing protoplasts which are capable to regenerate to whole plants.

With regard to the transformation of wheat various methods can be applied, e.g., agrobacterium-mediated gene transfer (Hiei et al., Plant J. 6 (1994), 271-282; Hiei et al., Plant Mol. Biol. 35 (1997), 205-218; Park et al., J. Plant Biol. 38 (1995), 365-371), protoplast transformation (Data in „Gene transfer to plants“, I. Potrykus, G. Spangenberg (Eds.), Springer-Verlag Berlin Heidelberg, 1995, pages 66-75; Datta et al., Plant Mol. Biol. 20 (1992), 619-629; Sadasivam et al., Plant Cell Rep. (1994), 394-396) the biolistic approach (Li et al., Plant Cell Rep. 12 (1993), 250-255; Cao et al., Plant Cell Rep. 11 (1992), 586-591; Christou, Plant Mol. Biol. 8 (1997), 197-203) and electroporation (Xu et al., in „Gene transfer to plants“, I. Potrykus, G. Spangenberg (Eds.), Springer-Verlag Berlin Heidelberg (1995), 201-208).

Once the introduced DNA has been integrated in the genome of the plant cell, it is usually stably integrated and remains within the genome of the descendants of the originally transformed cell. Usually the transformed cell contains a selectable marker gene which allows for the selections of the transformants in the presence of certain sugars, amino acids, biocides or antibiotics, e.g., kanamycin, G 428, bleomycin, hygromycin or phosphinothricine. Therefore, an individual marker gene allows for the selection of the transformed cells against cells lacking the introduced DNA.

After selection the transformed cells are cultivated under normal conditions and grow to a whole plant (McCormick et al., Plant Cell Reports 5 (1986), 81-84). The resulting plants can be cross-bred with plants having the same transformed genetic heritage or a different genetic heritage. Resulting individuals or hybrids have the corresponding phenotypic properties. Two or more generations should be grown in order to ensure whether the phenotypic feature is stable and transferable. Furthermore, seeds should be harvested in order to ensure that the corresponding phenotype or other properties will remain.

The modified starch obtainable from the plant cells, from the plants of the invention, or obtainable by the process of the invention is suitable for numerous industrial applications. Basically, starch can be subdivided into two major fields. One field comprises the hydrolysis products of starch, another the so-called native starches. The hydrolysis essentially comprise glucose and glucane components obtained by enzymatic or chemical processes. They can be used for further processes, such as fermentations and chemical modifications. Currently, starch hydrolysis is carried out substantially enzymatically using amyloglucosidase. Costs might be reduced by using lower amounts of enzyme for hydrolysis due to changes in the starch structure, e.g., increasing the surface of the grain, improved digestibility due to less branching or an altered steric structure, which limits the accesibility for the used enzymes. The use of the so-called native starch can be subdivided into the following areas:

(a) Use for the preparation of foodstuffs

Starch is a classic additive for various foodstuffs, wherein it essentially serves the purpose of binding aqueous additives and/or causes an increased viscositiy or an increased gel formation. Important characteristic properties are flowing and sorption behaviour, swelling and pastification temperature, viscositiy and thickening performance, solubility of the starch, transparency and paste structure, heat, shear and acid resistance, tendency to retrogradation, capability of film formation, resistance of freezing/thawing, digestibility as well as the capability of complex formation with , e.g., inorganic or organic ions.

(b) Use for the preparation of non-foodstuffs

The other major field of application is the use of starch as an adjuvant in various production processes or as an additive in technical products. The major fields of application for the use of starch as an adjuvant are, first of all, the paper and cardboard industry. In this field, the starch is mainly and for retention (holding back solids), for sizing filler and fine particles, as solidifying substance and for dehydration. In addition, the advantageous properties of starch with regard to

stiffness, hardness, sound, grip, gloss, smoothness, tear strength as well as the surfaces are utilized.

Within the paper production process, a differentiation can be made between four fields of application, namely surface, coating, mass and spraying.

The requirement on starch with regard to surface treatment are essentially a high degree of brightness, corresponding viscosity, high viscosity stability, good film formation as well as low formation of dust. When used in coating the solid content, a corresponding viscosity, a high capability to bind as well as high pigment affinity play an important role. As an additive to the mass rapid, uniform, loss-free dispersion, high mechanical stability and complete retention in the paper pulp are of importance. When using the starch in spraying, corresponding content of solids, high viscosity as well as high capability to bind are also significant. A major field of application is, for instance, in the adhesive industry, where the fields of application are subdivided into four areas: the use as pure starch glue, the use in starch glues prepared with special chemicals, the use of starch as an additive to synthetic resins and polymer dispersions as well as the use of starches as extenders for synthetic adhesives. 90 % of all starch-based adhesives are used in the production of corrugated board, paper sacks and bags, composites materials for paper and aluminium, boxes and wetting glue for envelopes, stamps, etc..

Another possible use as adjuvant and additive is in the production of textiles care products. Within the textile industry, a differentiation can be made between the following four fields of application: the use of starch as a sizing agent, i.e. as an adjuvant for smoothing and strengthening the burring behaviour for the protection against tensile forces active in weaving as well as for the increase of wear resistance during weaving as an agent for textile improvement mainly after quality-deteriorating pretreatments, such as bleaching dying, etc., as thickener in the production of dye pastes for the prevention of dye diffusion and as an additive for warping agents for sewing yarns.

Furthermore starch may be used as an additive in building materials. One example



is the production of gypsum plaster boards, wherein the starch mixed in the thin plaster pastifies with the water, diffuses at the surface of the gypsum board and thus binds the cardboard to the board. Other fields of application are admixing it to plaster and mineral fibers. In ready-mixed concrete, starch may be used for the deceleration of the sizing process.

Furthermore, the starch is advantageous for the production of means for ground stabilization used for the temporary protection of ground particles against water in artificial earth shifting. According to state-of-the-art knowledge, combination products consisting of starch and polymer emulsions can be considered to have the same erosion- and encrustation-reducing effect as the products used so far; however, they are considerably less expensive.

Another field of application is the use of starch in plant protectives for the modification of the specific properties of these preparations. For instance, starches are used for improving the wetting of plant protective and fertilisers, for the dosed release of the active ingredients, for the conversion of liquid, volatile and/or odorous active ingredients into microcrystalline, stable, deformable substances, for mixing incompatible compositions and for the prolongation of the duration of the effect due to a reduced disintegration.

Starch may also be used in the fields of drugs, medicine and in the cosmetics industry. In the pharmaceutical industry, the starch may be used as a binder for tablets or for the dilution of the binder in capsules. Furthermore, starch is suitable as disintegrant for tablets since, upon swallowing, it absorbs fluid and after a short time it swells so much that the active ingredient is released. It is also a suitable auxiliary to achieve a time-delayed release of the active ingredient (retardation effect). For qualitative reasons, medicinal flowance and dusting powders are further fields of application. In the field of cosmetics, the starch may for example be used as a carrier of powder additives, such as scents and salicylic acid. A relatively extensive field of application for the starch is toothpaste.



The use of starch as an additive in coal and briquettes is also suitable. By adding starch, coal can be quantitatively agglomerated and/or briquetted in high quality, thus preventing premature disintegration of the briquettes. Barbecue coal contains between 4 and 6 % added starch, calorated coal between 0.1 and 0.5 %.

Furthermore, the starch is suitable as a binding agent since adding it to coal and briquette can considerably reduce the emission of toxic substances.

Furthermore, the starch may be used as a flocculant in the processing of ore and coal slurry.

Another field of application is the use as an additive to process materials in casting. For various casting processes cores produced from sands mixed with binding agents are needed. Nowadays, the most commonly used binding agent is bentonite mixed with modified starches, mostly swelling starches.

The purposes of adding starch is increased flow resistance as well as improved binding strength. Moreover, swelling starches may fulfill more prerequisites for the production process, such as dispersability in cold water, rehydratisability, good mixability in sand and high capability of binding water.

In the rubber industry starch may be used for improving the technical and optical quality. Reasons for this are improved surface gloss, grip and appearance. For this purpose, the starch is dispersed on the stickly rubberized surfaces of rubber substances before the cold vulcanization. It may also be used for improving the printability of rubber.

Another field of application for the modified starch is the production of leather substitutes.

In the plastics market the following fields of application are emerging: the integration of products derived from starch into the processing process (starch is only a filler, there is no direct bond between synthetic polymer and starch) or, alternatively, the

integration of products derived from starch into the production of polymers (starch and polymer form a stable bond).

The use of the starch as a pure filler cannot compete with other substances such as talcum. This situation is different when the specific starch properties become effective and the property profile of the end products is thus clearly changed. One example is the use of starch products in the processing of thermoplastic materials, such as polyethylene. Thereby, starch and the synthetic polymer are combined in a ratio of 1:1 by means of coexpression to form a master batch, from which various products are produced by means of common techniques using granulated polyethylene. The integration of starch in polyethylene films may cause an increased substance permeability in hollow bodies, improved water vapor permeability, improved antistatic behaviour, improved antiblock behaviour as well as improved printability with aqueous dyes.

Another possibility is the use of the starch in polyurethane foams. Due to the adaption of starch derivatives as well as due to the optimization of processing techniques, it is possible to specifically control the reaction between synthetic polymers and the starch's hydroxy groups. The results are polyurethane films having the following property profiles due to the use of starch: a reduced coefficient of thermal expansion, decreased shrinking behaviour, improved pressure/tension behaviour, increased water vapor permeability without a change in water acceptance, reduced flammability and cracking density, no drop off of combustible parts, no halides and reduced aging. Disadvantages that presently still exists are reduced pressure and impact strength.

Product development of film is not the only option. Also solid plastics products, such as pots, plates and bowls can be produced by means of a starch content of more than 50 %. Furthermore, the starch/polymer mixtures offer the advantage that they are much easier biodegradable.

Furthermore, due to their extreme capability to bind water, starch graft polymers have gained utmost importance. These are products having a backbone of starch and a side lattice of a synthetic monomer grafted on according to the principle of radical chain mechanism. The starch graft polymers available nowadays are characterized by an improved binding and retaining capability of up to 1000 g water per g starch at a high viscosity. These super absorbers are used mainly in the hygiene field, e.g., in products such as diapers and sheets, as well as in the agricultural sector, e.g., in seed pellets.

#### Deposit of biological material

The following plasmids as described in the present invention were deposited in accordance with the requirements of the Budapest Treaty at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ) in Braunschweig, Germany :

Plasmid pTaR1-11 refers to accession number DSM No. 12810 at May 20, 1999.

Plasmid RS26-88 refers to accession number DSM No. 13511 at May 24, 2000.

The following Examples shall merely illustrate the invention and do not limit the invention in any way.

#### Example 1: Preparation of a cDNA from *Triticum aestivum* L., cv Florida encoding R1-protein

For identification and isolation of a cDNA encoding R1-protein derived from wheat a wheat cDNA library was prepared from poly(A)<sup>+</sup>RNA of a 21 day old caryopsis ("starchy"-endosperm) of wheat plants by use of lambda zap II vector (Lambda ZAP II-cDNA Synthesis Kit, Stratagene GmbH, Heidelberg, Germany) according to the manufacturer's protocol. The primary titer of the cDNA library was about  $1,26 \times 10^6$  pfu/ml.

Screening of the cDNA library was performed using the oligonucleotides R1A and R1B as primers for PCR (polymerase chain reaction) amplification of a DNA insert of plasmid pBinAR Hyg (DSM 9505) containing a cDNA encoding R1-protein derived from maize. Said plasmid is, e.g., obtainable according to Example 14 of WO 98/27212. Therefore, the disclosure content of WO 98/27212-A1 is expressly incorporated herein by reference.

After Xba I/Asp 718 restriction endonuclease digestion of vector pBluescript, a cDNA fragment was purified by agarose gel electrophoresis and standard protocols.

As a template for the PCR-amplification of said maize cDNA fragment, about 10 pg of the above isolated maize cDNA fragment were used.

The PCR buffer contained 1.5mM MgCl<sub>2</sub>, 20mM Tris-HCL (pH 8.4), 50mM KCl, 0.8mM dNTP mix, 1µM primer R1A, 1µM primer R1B und 2.5 units Taq polymerase.  
R1A: 5' TATTGGAAGCTCGAGTTGAAC 3' (Seq. ID No. 3)  
R1B: 5' TTGAGCTGTCTAATAGATGCA 3' (Seq. ID No. 4)

PCR cycling was performed in a Trioblock<sup>®</sup> PCR-thermocycler (Biometra, Germany) according to the following protocol: 4' at 95°C; 1' at 96°C; 45' ' at 62°C; 1' 15' ' at 72°C; 30 cycles and 5' at 72°C in order to amplify a cDNA fragment encoding R1-protein derived from maize.

Subsequently, the obtained fragment was random-primed digoxigenin-labelled according to the manufacturers protocol (Boehringer Mannheim, The DIG system users Guide).

The amplified and labelled cDNA fragment of 1924 bp was further used as a heterologous probe for the screening of the above prepared cDNA library derived from wheat.

About  $3.5 \times 10^5$  phages were screened according to standard protocols.

After pre-hybridization in 5 x SSC, 3% Blocking (Boehringer Mannheim), 0.2% SDS, 0.1% sodium laurylsarcosine and 50 µg/ml herring sperm DNA at 55°C, the filters were hybridized overnight with 1 ng/ml of the digoxigenin labeled (Random Primed DNA Labeling Kit) r1-protein probe (the 1924 bp XbaI/Asp718 cDNA fragment of maize). The filters were washed 2 times for 5' with 2X SSC, 1% SDS at room temperature; 2 times for 10' with 1X SSC, 0.5% SDS at 55°C, and 2 times for 10' in 0.5X SSC, 0.2% SDS at 55°C.

Positive clones were rescreened and purified. The plasmids (pBluescript SK Phagemide) were isolated by *in vivo excision*, according to the manufacturer's protocol (Stratagene, Heidelberg). After characterization of the clones by restriction analysis, the longest cDNA inserts were further analyzed.

#### Example 2: Sequence analysis of cDNA insert of pTaR1-11

The nucleotide sequence of the isolated cDNA insert of clone pTaR1-11 was analyzed according to the dideoxynucleotide method (Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977), 5463-5467).

Clone TaR1-11 contains a 672bp insert representing a partial cDNA according to Seq. ID No. 1 encoding R1-protein according to Seq. ID No. 2 derived from wheat.

The corresponding amino acid sequence of the polynucleotide of Seq ID No. 1 is given in Seq. ID. No. 2.

#### Example 3: Isolation and sequence identification of a cDNA from *Triticum aestivum* L. cv Florida encoding R1-protein

For identification and isolation of a cDNA encoding the R1-protein from wheat poly(A<sup>+</sup>)-RNA was isolated from 3-6 weeks old leaves from wheat and reverse transcribed using RT-PCR-Kit (Titan One tube RT-PCR System, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Amplification of a cDNA encoding the R1-protein was performed using oligo-nucleotides Zm-R1-2 (Seq ID No. 5) and Wh-R1-5 (Seq ID No. 6) and an aliquot of RT-reaction as template.

The following primers were selected as hybridisation probe for the isolation of the desired DNA encoding R1 protein: The primer binding sites are localized in Seq. ID no's. 7 and 9 at position 1-24 and 3402-3418):

Zm-R1-2 (Seq ID No. 5):

5'- CTG TGG TCT TGT CTG GAC-3'

Wh-R1-5' (Seq ID No. 6):

5'-GAG GAA GCA AGG AAG GAA CTG CAG-3'

The PCR-reaction was performed in an Eppendorf Mastercycler™ gradient (Eppendorf, Hamburg, Germany) and contained 10 mM Tris-HCl pH 8,85, 25 mM KCl, 5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1,5 mM MgSO<sub>4</sub>, 0,8 mM dNTPs, 1 µM Primer Zm-R1-2, 1 µM Primer Wh-R1-5 and 1 Unit Pwo-DNA- Polymerase. The following temperature program was proceeded:

Initially 2' at 94°C, then 35 cycles of 1' 94°C, 1' at 55°C and 3' at 72°C and a final step of 5' at 72°C. The obtained DNA-fragment of 3,4 kb was cloned into the EcoRV-site of a pBluescript SK(-) vector resulting in plasmid RS 23-88 was further analysed for the nucleotide sequence in cooperation with GATC GmbH (Konstanz, Germany) and specified as SEQ ID No. 7. represents the main-part of the R1-gene with ~1kb of the 5'-end and ~300 bp of the 3'-end lacking. The missing 3'-region was complemented with the corresponding region of a partial R1-cDNA clone as described in example 1 and 2 resulting in plasmid RS 26-88 and comprising SEQ ID No. 9. In order to achieve that the clone RS 23-88 was digested with the restriction



endonuclease *Ec*/136. The resulting large fragment was used for further cloning, whereas the smaller 140bp fragment was discarded. The clone TaR1-11 from example 1 and 2 which contains the 3'-region of the R1 cDNA from wheat was treated with the restriction endonuclease *Xho*I, the restriction site was filled up to blunt end using T4-DNA-Polymerase and the 3'-region of the R1 cDNA from wheat was released from the vector by digestion with the restriction endonuclease *Ec*/136. This produced fragment was ligated to the blunt ends of *Ec*/136-digested RS 23-88. The orientation of the ligated fragment was controlled by restriction analysis. The primary structure of the whole cDNA clone (~3,7 kb) was again determined by sequence analysis performed by GATC GmbH (Konstanz, Germany) and specified as SEQ ID No. 9.

## Claims:

1. A nucleic acid molecule encoding an R1-protein or part or derivative of R1-protein selected from the group consisting of:
  - (a) a nucleic acid molecule encoding an R1 protein comprising a polypeptide selected from the group consisting of Seq. ID No. 2 and Seq. ID No. 10 or part or derivative thereof;
  - (b) a nucleic acid molecule selected from the group consisting of Seq. ID No. 1 and Seq. ID No. 9 or part or derivative thereof;
  - (c) a nucleic acid molecule comprising the coding region of the cDNA insert selected from the group consisting of plasmid pTa R1-11 according to DSM No.12810 and plasmid RS26-88 according to DSM No.13511 or part or derivative thereof;
  - (d) a nucleic acid molecule encoding a polypeptide comprising the polypeptide encoded by the cDNA insert selected from the group consisting of plasmid pTa R1-11 according to DSM No. 12810 and plasmid RS26-88 according to DSM No.13511 or part or derivative thereof.
2. A nucleic acid molecule according to claim 1 comprising one or more regulatory elements which ensure transcription and/or translation in a cell.
3. A nucleic acid molecule according to one or more of claims 1 to 2, which is a DNA molecule.
4. A nucleic acid molecule according to one or more of claims 1 to 2, which is an RNA molecule.
5. A vector comprising a nucleic acid molecule according to one or more of claims 1 to 4.
6. The vector according to claim 5, comprising one or more regulatory elements

which ensures transcription and/or translation in a bacterial and/or a plant cell.

7. A transgenic host cell, comprising a nucleic acid molecule according to one or more of claims 1 to 4 and/or a vector according to one or more of claims 5 to 6.
8. The host cell according to claim 7, which is a plant cell.
9. A process for the preparation of a transgenic cell according to one or more of claims 7 to 8 comprising the step of introducing a nucleic acid molecule according to one or more of claims 1 to 4 and/or a vector according to one or more of claims 5 to 6 into the genome of a procaryotic or eucaryotic cell.
10. The process according to claim 9 wherein said cell is a plant cell.
11. A transgenic plant comprising the host cell according to claim 8.
12. A process for the production of a plant according to claim 11 comprising the steps of introducing into a plant cell a nucleic acid molecule according to one or more of claims 1 to 4 and/or a vector according to one or more of claims 5 to 6 and regenerating a whole plant from the plant cell.
13. A propagation material from the plant according to claim 11.
14. A seed from the transgenic plant according to claim 11.
15. A process for the production of a starch comprising the step of introducing a plant cell according to claim 8, a plant according to claim 11, a propagation material according to claim 13 and/or a seed according to claim 14 into a process for the production of starch.

16. A starch obtainable from a plant cell according to claim 8, a plant according to claim 11, a propagation material according to claim 13 and/or a seed according to claim 14 or by the process according to claim 15.
17. A process for the production of a modified starch comprising the step of introducing a starch according to claim 16 into a process of chemical and/or physical modification of starch.
18. A process for the production of an R1-polypeptide according to Seq. ID No. 2 and/or Seq. ID No. 10 or derivatives or parts thereof comprising the steps of cultivating the host cell according to one or more of claims 7 to 8 under conditions allowing for the expression of the protein and isolating said R1-polypeptide from said cells and/or the culture medium.
19. An R1-polypeptide encoded by the nucleic acid molecule according to one or more of claims 1 to 4 or derivative or part thereof.
20. The use of an R1-polypeptide according to claim 19 for the production of a monoclonal or polyclonal antibody.
21. The use of a nucleic acid molecule or derivative or part thereof according to one or more of claims 1 to 4 for the screening of nucleic acid libraries and/or as a probe for hybridisation, said nucleic acid molecule or derivative or part thereof being optionally labelled.
22. The use of a nucleic acid molecule or derivative or part thereof according to one or more of claims 1 to 4 for the preparation of a transgenic cell or transgenic plant.

## SEQUENCE LISTING

<110> Aventis CropScience GmbH

<120> Nucleic acid molecules from wheat, transgenic plant cells and plants and the use thereof for the production of modified starch

<130> AGR 1999/M 214

<150> DE 199 26 771.5

<151> 1999-06-11

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Leu Gly Glu Thr Leu Val Gly Ala Tyr Pro Gly Arg Ala Met Ser Phe	
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Ser Lys Pro Ile Gly Leu Phe Ile Lys Arg Ser Ile Ile Phe Arg Ser	
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Lys	Ala	Cys	Glu	Thr	Ser	Phe	Gly	Glu	Ser	Glu	Leu	Asp	Gly	Leu	Gln
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10

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Ile	Gln	Met	Leu	Lys	Gly	Arg	Leu	Ala	Gln	Glu	Asp	Phe	Ser	Ala	Leu
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11

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Lys Val Trp Ala Ser Lys Trp Asn Glu Arg Ala Tyr Phe Ser Thr Arg  
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Trp Glu Ala Pro Pro Ser Ser Ile Val Pro Ser Gly Ser Thr Val Leu	
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13

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Val Thr Tyr Arg Glu Val Ser Asp Ser Glu Leu Met Gln Ser Ser Ser	
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Ser Asp Ala Gln Gly Gly Glu Ala Ile Pro Ser Leu Ser Leu Val Lys	
800 805 810 815	
aaa aag ttc ctt gga aaa tat gca ata tca gcg gaa gag ttc tct gat	2495
Lys Lys Phe Leu Gly Lys Tyr Ala Ile Ser Ala Glu Glu Phe Ser Asp	
820 825 830	
gaa atg gtt gga gca aag tcc cgc aac ata gca tac ctg aaa gga aaa	2543
Glu Met Val Gly Ala Lys Ser Arg Asn Ile Ala Tyr Leu Lys Gly Lys	
835 840 845	
gta cct tca tgg gtt ggt atc cca aca tca gtt gcg ata cca ttt ggg	2591
Val Pro Ser Trp Val Gly Ile Pro Thr Ser Val Ala Ile Pro Phe Gly	
850 855 860	
acc ttt gag aag ata ttg tct gat gag acc aat aag gaa gta gca caa	2639
Thr Phe Glu Lys Ile Leu Ser Asp Glu Thr Asn Lys Glu Val Ala Gln	
865 870 875	
aac ata cag atg ctg aag ggc aga ctt gct caa gaa gat ttt agt gct	2687
Asn Ile Gln Met Leu Lys Gly Arg Leu Ala Gln Glu Asp Phe Ser Ala	
880 885 890 895	
cta gga gaa atc cgg aaa act gtt ctt aat cta act gct cca act caa	2735
Leu Gly Glu Ile Arg Lys Thr Val Leu Asn Leu Thr Ala Pro Thr Gln	
900 905 910	
ccg gtt aag gag ctg aag gag aag atg cta agc tcc gga atg ccc tgg	2783
Pro Val Lys Glu Leu Lys Glu Lys Met Leu Ser Ser Gly Met Pro Trp	
915 920 925	
cct gga gat gaa agt gac cac cgt tgg gag caa gca tgg atg gca att	2831
Pro Gly Asp Glu Ser Asp His Arg Trp Glu Gln Ala Trp Met Ala Ile	
930 935 940	
aaa aag gtt tgg gca tca aaa tgg aat gaa aga gca tac ttt agt aca	2879
Lys Lys Val Trp Ala Ser Lys Trp Asn Glu Arg Ala Tyr Phe Ser Thr	
945 950 955	
cgc aag gtg aag ctc gat cat gag tac ctt tcc atg gct gtt ctt gta	2927
Arg Lys Val Lys Leu Asp His Glu Tyr Leu Ser Met Ala Val Leu Val	
960 965 970 975	
caa gaa att gtc aac gca gac tat gcc ttt gtc att cat act acg aac	2975
Gln Glu Ile Val Asn Ala Asp Tyr Ala Phe Val Ile His Thr Thr Asn	
980 985 990	
ccg tca tct gga gat tct tct gag ata tat gct gaa gtg gtg aaa gga	3023
Pro Ser Ser Gly Asp Ser Ser Glu Ile Tyr Ala Glu Val Val Lys Gly	
995 1000 1005	

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ctt gga gag aca ctt gtg gga gct tat cct ggc cgt gcc atg agc ttc 3071  
Leu Gly Glu Thr Leu Val Gly Ala Tyr Pro Gly Arg Ala Met Ser Phe  
1010 1015 1020

gtg tgt aag aaa gat gac ctt gac tct ccc aag gta ctg ggt tac cct 3119  
Val Cys Lys Lys Asp Asp Leu Asp Ser Pro Lys Val Leu Gly Tyr Pro  
1025 1030 1035

agc aag cca att ggt ctc ttc ata aag cgg tca atc atc ttc cgc tca 3167  
Ser Lys Pro Ile Gly Leu Phe Ile Lys Arg Ser Ile Ile Phe Arg Ser  
1040 1045 1050 1055

gac tct aat ggt gag gat ctg gaa ggt tac gct gga gca ggg ctg tat 3215  
Asp Ser Asn Gly Glu Asp Leu Glu Gly Tyr Ala Gly Ala Gly Leu Tyr  
1060 1065 1070

gat agt gtc cct atg gat gtg gaa gat gaa gtt gta ctc gac tac acg 3263  
Asp Ser Val Pro Met Asp Val Glu Asp Glu Val Val Leu Asp Tyr Thr  
1075 1080 1085

acc gac cct ctc atc act gac tct gga ttc cgg aac tca atc ctc tca 3311  
Thr Asp Pro Leu Ile Thr Asp Ser Gly Phe Arg Asn Ser Ile Leu Ser  
1090 1095 1100

agc att gca cgg gct ggc cac gcc atc gag gag ctc tat ggg tca cca 3359  
Ser Ile Ala Arg Ala Gly His Ala Ile Glu Glu Leu Tyr Gly Ser Pro  
1105 1110 1115

cag gat gtt gag gga gta gtg aag gat ggg aag atc tac gta gtc cag 3407  
Gln Asp Val Glu Gly Val Val Lys Asp Gly Lys Ile Tyr Val Val Gln  
1120 1125 1130 1135

aca tac cac aga tgt aat atg tat gta tac gcg gct caa gtt gta gag 3455  
Thr Tyr His Arg Cys Asn Met Tyr Val Tyr Ala Ala Gln Val Val Glu  
1140 1145 1150

tag taggatatat ggtccttgct ggcatgtata gttgtactca taggtgcaca 3508

acacatctac gttgttattt atttgcatac acgctcagaa taagctttga tcacatactg 3568

tatttcctag agtaccagaa agtgtatgta cgatcaggaa tatgacctta ttaaaaccat 3628

tgaggggaaa tgttttgact tttgagcaat ctaaaaaaaa aaaaaaaaaa 3678

&lt;210&gt; 10

&lt;211&gt; 1152

&lt;212&gt; PRT

&lt;213&gt; Triticum aestivum

&lt;400&gt; 10

Gly Arg Arg Lys Glu Leu Gln Ala Glu Leu Asp Asn Gly Ala Ser Val  
1 5 10 15

Asp Gln Leu Arg Lys Lys Ile Val Lys Gly Asn Leu Glu Lys Lys Val  
20 25 30

Ser Lys Gln Leu Glu Lys Lys Lys Tyr Phe Ser Val Glu Arg Ile Gln  
35 40 45

Arg Arg Asn Arg Asp Ile Thr Gln Leu Leu Asn Lys His Lys Pro Val  
50 55 60

Val	Thr	Glu	Gln	Gln	Val	Lys	Ala	Ala	Pro	Lys	Gln	Pro	Thr	Val	Leu	65	70	75	80
Asp	Leu	Phe	Thr	Lys	Ser	Leu	Gln	Glu	Gly	Asp	Asn	Cys	Asp	Val	Leu	85	90	95	
Ser	Arg	Lys	Leu	Phe	Lys	Ile	Gly	Asp	Glu	Glu	Ile	Leu	Ala	Ile	Ala	100	105	110	
Thr	Asn	Ala	Leu	Gly	Lys	Thr	Arg	Val	His	Leu	Ala	Thr	Asn	Arg	Met	115	120	125	
Glu	Pro	Leu	Ile	Leu	His	Trp	Ala	Leu	Ala	Lys	Asn	Pro	Gly	Glu	Trp	130	135	140	
Glu	Ala	Pro	Pro	Ser	Ser	Ile	Val	Pro	Ser	Gly	Ser	Thr	Val	Leu	Asp	145	150	155	160
Lys	Ala	Cys	Glu	Thr	Ser	Phe	Gly	Glu	Ser	Glu	Leu	Asp	Gly	Leu	Gln	165	170	175	
Tyr	Gln	Val	Val	Glu	Ile	Glu	Leu	Asp	Asp	Gly	Arg	Tyr	Lys	Gly	Met	180	185	190	
Pro	Phe	Val	Leu	Arg	Arg	Gly	Glu	Thr	Trp	Ile	Lys	Asn	Asn	Asp	Ser	195	200	205	
Asp	Phe	Tyr	Leu	Asp	Phe	Asn	Thr	Lys	Val	Thr	Lys	Lys	Ser	Lys	Asp	210	215	220	
Thr	Gly	Asp	Ala	Gly	Lys	Gly	Thr	Ala	Lys	Asp	Phe	Leu	Glu	Arg	Ile	225	230	235	240
Ala	Asp	Leu	Glu	Glu	Asp	Ala	Gln	Arg	Ser	Phe	Met	His	Arg	Phe	Asn	245	250	255	
Ile	Ala	Ala	Asp	Leu	Val	Asp	Gln	Ala	Arg	Asp	Ala	Gly	Leu	Leu	Gly	260	265	270	
Ile	Val	Gly	Leu	Phe	Val	Trp	Ile	Arg	Phe	Met	Ser	Thr	Arg	Gln	Leu	275	280	285	
Ile	Trp	Asn	Lys	Asn	Tyr	Asn	Val	Lys	Pro	Arg	Glu	Ile	Ser	Gln	Ala	290	295	300	
Gln	Asp	Arg	Phe	Thr	Asp	Asp	Leu	Glu	Asn	Met	Tyr	Lys	Ser	Tyr	Pro	305	310	315	320
Gln	Tyr	Arg	Glu	Ile	Leu	Arg	Met	Leu	Leu	Ser	Ala	Val	Gly	Arg	Gly	325	330	335	
Gly	Glu	Gly	Asp	Val	Gly	Gln	Arg	Ile	Arg	Asp	Glu	Ile	Leu	Val	Ile	340	345	350	
Gln	Arg	Asn	Asn	Asp	Cys	Lys	Gly	Gly	Ile	Met	Glu	Glu	Trp	His	Gln	355	360	365	
Lys	Leu	His	Asn	Asn	Thr	Ser	Pro	Asp	Asp	Val	Val	Ile	Cys	Gln	Ala	370	375	380	
Ile	Ile	Asp	Tyr	Ile	Lys	Ser	Asp	Phe	Asp	Ile	Asn	Val	Tyr	Trp	Asp	385	390	395	400

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Thr Leu Asn Lys Asn Gly Ile Thr Lys Glu Arg Leu Leu Ser Tyr Asp  
 405 410 415  
 Arg Ala Ile His Ser Glu Pro Lys Phe Arg Ser Asp Gln Lys Glu Gly  
 420 425 430  
 Leu Leu Arg Asp Leu Gly Asn Tyr Met Arg Ser Leu Lys Ala Val His  
 435 440 445  
 Ser Gly Ala Asp Leu Glu Ser Ala Ile Ala Thr Cys Met Gly Tyr Lys  
 450 455 460  
 Ser Glu Gly Glu Gly Phe Met Val Gly Val Gln Ile Asn Pro Val Asn  
 465 470 475 480  
 Gly Leu Ser Ser Gly Phe Pro Asp Leu Leu Gln Phe Val Leu Asp His  
 485 490 495  
 Val Glu Asp Lys Ser Ala Glu Pro Leu Leu Glu Gly Leu Leu Glu Ala  
 500 505 510  
 Arg Val Glu Leu Arg Pro Leu Leu Thr Gly Ser Ser Glu Arg Leu Lys  
 515 520 525  
 Asp Leu Ile Phe Leu Asp Ile Ala Leu Asp Ser Thr Phe Arg Thr Ala  
 530 535 540  
 Val Glu Arg Ser Tyr Glu Glu Leu Asn Asp Ala Ala Pro Glu Lys Ile  
 545 550 555 560  
 Met Tyr Phe Ile Ser Leu Val Leu Glu Asn Leu Ala Leu Ser Thr Asp  
 565 570 575  
 Asp Asn Glu Asp Ile Leu Tyr Cys Leu Lys Gly Trp Asn Arg Ala Met  
 580 585 590  
 Asp Met Val Lys Gln Lys Asp Asp Gln Trp Ala Leu Tyr Ala Lys Ala  
 595 600 605  
 Phe Leu Asp Arg Thr Arg Leu Ala Leu Ala Ser Lys Gly Glu Gln Tyr  
 610 615 620  
 Tyr Asn Met Met Gln Pro Ser Ala Glu Tyr Leu Gly Ser Leu Leu Asn  
 625 630 635 640  
 Val Glu Glu Trp Ala Val Asp Ile Phe Thr Glu Glu Val Ile Arg Gly  
 645 650 655  
 Gly Ser Ala Ala Thr Leu Ser Ala Leu Leu Asn Arg Phe Asp Pro Val  
 660 665 670  
 Leu Arg Asn Val Ala His Leu Gly Ser Trp Gln Val Ile Ser Pro Val  
 675 680 685  
 Glu Val Thr Gly Tyr Ile Val Val Val Asp Lys Leu Leu Ser Val Gln  
 690 695 700  
 Asn Lys Thr Tyr Asp Lys Pro Thr Ile Leu Val Ala Lys Ser Val Lys  
 705 710 715 720  
 Gly Glu Glu Glu Ile Pro Asp Gly Val Val Gly Val Ile Thr Pro Asp  
 725 730 735



Met Pro Asp Val Leu Ser His Val Ser Val Arg Ala Arg Asn Cys Lys  
 740 745 750  
 Val Leu Phe Ala Thr Cys Phe Asp Pro Asn Thr Leu Ser Glu Phe Gln  
 755 760 765  
 Gly His Glu Gly Lys Val Phe Ser Phe Lys Thr Thr Ser Ala Asp Val  
 770 775 780  
 Thr Tyr Arg Glu Val Ser Asp Ser Glu Leu Met Gln Ser Ser Ser Ser  
 785 790 795 800  
 Asp Ala Gln Gly Gly Glu Ala Ile Pro Ser Leu Ser Leu Val Lys Lys  
 805 810 815  
 Lys Phe Leu Gly Lys Tyr Ala Ile Ser Ala Glu Glu Phe Ser Asp Glu  
 820 825 830  
 Met Val Gly Ala Lys Ser Arg Asn Ile Ala Tyr Leu Lys Gly Lys Val  
 835 840 845  
 Pro Ser Trp Val Gly Ile Pro Thr Ser Val Ala Ile Pro Phe Gly Thr  
 850 855 860  
 Phe Glu Lys Ile Leu Ser Asp Glu Thr Asn Lys Glu Val Ala Gln Asn  
 865 870 875 880  
 Ile Gln Met Leu Lys Gly Arg Leu Ala Gln Glu Asp Phe Ser Ala Leu  
 885 890 895  
 Gly Glu Ile Arg Lys Thr Val Leu Asn Leu Thr Ala Pro Thr Gln Pro  
 900 905 910  
 Val Lys Glu Leu Lys Glu Lys Met Leu Ser Ser Gly Met Pro Trp Pro  
 915 920 925  
 Gly Asp Glu Ser Asp His Arg Trp Glu Gln Ala Trp Met Ala Ile Lys  
 930 935 940  
 Lys Val Trp Ala Ser Lys Trp Asn Glu Arg Ala Tyr Phe Ser Thr Arg  
 945 950 955 960  
 Lys Val Lys Leu Asp His Glu Tyr Leu Ser Met Ala Val Leu Val Gln  
 965 970 975  
 Glu Ile Val Asn Ala Asp Tyr Ala Phe Val Ile His Thr Thr Asn Pro  
 980 985 990  
 Ser Ser Gly Asp Ser Ser Glu Ile Tyr Ala Glu Val Val Lys Gly Leu  
 995 1000 1005  
 Gly Glu Thr Leu Val Gly Ala Tyr Pro Gly Arg Ala Met Ser Phe Val  
 1010 1015 1020  
 Cys Lys Lys Asp Asp Leu Asp Ser Pro Lys Val Leu Gly Tyr Pro Ser  
 025 1030 1035 1040  
 Lys Pro Ile Gly Leu Phe Ile Lys Arg Ser Ile Ile Phe Arg Ser Asp  
 1045 1050 1055  
 Ser Asn Gly Glu Asp Leu Glu Gly Tyr Ala Gly Ala Gly Leu Tyr Asp  
 1060 1065 1070

20

Ser Val Pro Met Asp Val Glu Asp Glu Val Val Leu Asp Tyr Thr Thr  
1075 1080 1085

Asp Pro Leu Ile Thr Asp Ser Gly Phe Arg Asn Ser Ile Leu Ser Ser  
1090 1095 1100

Ile Ala Arg Ala Gly His Ala Ile Glu Glu Leu Tyr Gly Ser Pro Gln  
105 1110 1115 1120

Asp Val Glu Gly Val Val Lys Asp Gly Lys Ile Tyr Val Val Gln Thr  
1125 1130 1135

Tyr His Arg Cys Asn Met Tyr Val Tyr Ala Ala Gln Val Val Glu  
1140 1145 1150